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Post-translational processing and Thr-206 are required for glycosylasparaginase activity

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Lysosomal glycosylasparaginase is encoded as a 36.5 kDa polypeptide that is post-translationally processed to subunits of 19.5 kDa (heavy) and 15 kDa (light). Recombinant glycosylasparaginase has been expressed in *Spodoptera frugiperda* insect cells enabling the precursor and processed forms to be isolated and their catalytic potential determined. Only the subunit conformation was functional indicating glycosylasparaginase is encoded as an inactive zymogen. The newly created amino terminal residue of the light subunit following maturation, Thr-206, is believed to be involved in the catalytic mechanism [1992, J. Biol. Chem. 267, 6855–6858]. Here we have constructed two amino acid substitution mutants replacing Thr-206 with Ala-206 or Ser-206 and demonstrate that both destroy enzyme activity.

Glycosylasparaginase; Post-translational activation; Active-site mutagenesis

1. INTRODUCTION

Glycosylasparaginase (1-aspartamido-β-*N*-acetylglucosamine amidohydrolase, EC 3.5.1.26) is a lysosomal hydrolase that cleaves the β-*N*-aspartylglucosylamine bond of asparagine-linked glycoproteins [1]. This amidase has been biochemically characterized from various species and is composed of two non-identical subunits of approximately 25 kDa (heavy) and 19 kDa (light) that are held together by strong non-covalent forces [2,3]. Molecular cloning of the human glycosylasparaginase cDNA [4] and gene [5] sequences revealed the heavy and light subunits are derived from a common precursor polypeptide. Interestingly, this post-translational processing event appears to constitute an activation step. Our laboratory originally proposed glycosylasparaginase might be encoded as an inactive zymogen based on expression studies of a point mutation (G488 → C) discovered in the human glycosylasparaginase gene pool that is responsible for the high incidence of the lysosomal storage disorder aspartylglucosaminuria (AGU) in Finland [6–8]. This mutation results in a single amino acid substitution (Cys-163 → Ser) in the glycosylasparaginase heavy subunit that destroys catalytic activity and prevents post-translational processing of the precursor to the heavy-light subunit organization [8]. The mutant Finnish AGU polypeptide is additionally not transported to the lysosome, but instead appears trapped in the ER or other early compartment [9]. In a more recent study we characterized a point mutation from a British family with two AGU afflicted siblings that results in an Ala-101 → Val substitution in the glycosylasparaginase heavy subunit [10,11]. Expression studies revealed this amino acid substitution is phenotypically identical to the Finnish mutation in destroying catalytic activity, subunit processing, and lysosomal transport [11].

We first attempted to experimentally demonstrate a correlation between glycosylasparaginase subunit maturation and enzyme activity by testing the catalytic potential of the single-chain precursor generated from an in vitro expression system [8]. The protocol that was followed had been optimized to achieve native protein folding and was shown by Sonderfeld-Fresko and Proia to produce a functional β-*N*-acetyl-hexosaminidase enzyme [12]. Although the glycosylasparaginase precursor was unable to cleave the GlcNAc-Asn substrate and likely inactive, it was difficult to conclude that the translated product had indeed attained its native conformation. Without such assurances, the negative result could have been due to improper folding as opposed to a protein lacking catalytic activity. In this study we selected the *Spodoptera frugiperda* insect cell–baculovirus expression system as an alternative for producing the single-chain precursor [13].

The importance of the post-translational cleavage event that generates the heavy and light subunits of glycosylasparaginase becomes more intriguing in light of a study by Kaartinen et al. [16]. These researchers showed that the hydroxyl group of Thr-206, which oc-

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cupies the amino terminal position of the newly formed light subunit, forms an α-ketone ether linkage with the reacting group of the asparagine analog diazo-4-oxo-L-norvaline (DONV). This compound has been shown to be a potent irreversible inhibitor of glycosylasparaginase and other amidohydrolases [17,18]. If Thr-206 is indeed involved in the catalytic mechanism of glycosylasparaginase as proposed by Kaartinen et al. [16], its shift from an endo-position in the precursor polypeptide to the amino terminus of the light subunit following proteolysis might have important implications for the catalytic mechanism. To address the role of Thr-206 at the glycosylasparaginase active center we have constructed two mutant cDNAs, each of which carries a different amino acid substitution at Thr-206, for expression in COS-1 cells.

2. MATERIALS AND METHODS

2.1. *Expression of glycosylasparaginase from a recombinant baculovirus*

A baculovirus expression vector, pVT-Bac, engineered to enhance secretion of heterologous proteins was kindly provided by Dr. Thierry Vernet (National Research Council of Canada, Montreal, Quebec) and has been described [19] The critical feature of pVT-Bac is the presence of sequence which encodes the signal peptide from honeybee melittin, a secreted protein, followed by a multiple cloning site (MCS). Insertion of a transgene into the MCS allows for the production of a fusion protein containing the secretion-targeted honeybee melittin signal peptide. Here we introduced a human placenta glycosylasparaginase cDNA lacking its signal peptide ($GSA^{\Delta Sig}$) into the MCS of pVT-Bac. The 5′ end of $GSA^{\Delta Sig}$ was created by the polymerase chain reaction with a sense-strand oligonucleotide, 5′ CT CTG CCC CTG GTC GTC A 3′, that aligned to bp positions 77–94 of the human placenta glycosylasparaginase cDNA [4]. The antisense-strand primer, 3′ AGAAATGACAGTTGTAGACA 5′, was according to bp positions 1,046–1,065 of the human placenta glycosylasparaginase cDNA 3′-untranslated region [4]. PCR reaction conditions were as described [8]. The $GSA^{\Delta Sig}$ PCR product was purified by agarose gel elution and cloned into a blunt-ended *Bam*HI site located within the MCS of pVT-Bac to create the plasmid pBac[mel-GSA]. This cloning strategy resulted in an amino acid deletion and a substitution at the amino terminus of the human glycosylasparaginase heavy subunit. Therefore, the predicted amino terminus following removal of the honeybee melittin signal peptide would read H_2N-DPLPLVVNT..., compared to H_2N-SSPLPLVVNT... after removal of the glycosylasparaginase signal peptide The entire sequence of the modified human mel-GSA cDNA as constructed in pBac[mel-GSA] was verified by DNA sequencing.

Recombinant baculovirus containing the mel-GSA fusion protein cDNA, Ac[mel-GSA], were produced by co-transfection of *Spodoptera frugiperda* Sf9 cells with pBac[mel-GSA] and linear wild-type virus AcMNPV (Clonetech) [20] Preparative expression of recombinant mel-GSA was performed in 100 ml spinner cultures of Sf9 cells infected with Ac[mel-GSA] [20]. Culture media and cells were harvested at 72 hours post-infection (hpi). A small volume of the culture was permitted to grow for an additional 48 h to establish a 120 hpi time point. Cells collected at 72 hpi were pelleted, washed, and an extract prepared by sonication. Cell debris was removed by centrifugation and the clarified supernatant applied to a 2.5 × 100 cm Sephacryl S-200 column at a flow rate of 0.25 ml/min Media and cell extract samples were analyzed by immunoblotting, assayed for glycosylasparaginase activity, and treated with *N*-glycanase (Genzyme) as described [8].

2.2. *Construction and expression of glycosylasparaginase active-site mutants*

Two glycosylasparaginase cDNAs, $GSA^{Ala\text{-}206}$ and $GSA^{Ser\text{-}206}$, representing two different amino acid substitutions at Thr-206 were constructed by oligonucleotide-directed mutagenesis mediated by the polymerase chain reaction. For each mutant a sense-strand oligonucleotide was synthesized that aligned to bp positions 605–622 of the human placenta glycosylasparaginase cDNA [4]. Within this 18 nucleotide stretch was the codon for Thr-206 and a unique *Bsp*HI site directly 5′ to the mutagenesis target site The oligonucleotides contained a single base change at the first position of the *ACT* codon encoding Thr-206 resulting in an Ala-206 substitution, 5′ GT GGT CAT GAC *GCT* ATT G 3′, or a Ser-206 substitution, 5′ GT GGT CAT GAC *TCT* ATT G 3′ Each sense-strand primer used a common antisense-strand oligonucleotide, 3′ AGAAATGAC-AGTTGTAGACA 5′, which aligned to bp positions 1,046–1,065 of the human placenta glycosylasparaginase cDNA 3′-untranslated region [4]. PCR reaction conditions were as described [8]. Amplification products were purified by agarose gel elution, digested with *Bsp*HI, and ligated with a *Bsp*HI fragment encoding the glycosylasparaginase heavy subunit The resulting full-length mutant glycosylasparaginase cDNA containing their respective amino acid substitution were verified by DNA sequencing.

$GSA^{Ala\text{-}206}$ and $GSA^{Ser\text{-}206}$ were independently subcloned into the expression vector pSVL (Pharmacia LKB) Transfection into COS-1 cells was by the DEAE-dextran method using 5 μg/100 mm culture dish of either recombinant or native pSVL [8]. Cells were harvested 48 h post-transfection and analyzed for the presence of recombinant protein and enzyme activity [8].

3. RESULTS AND DISCUSSION

3.1. *Expression of precursor and processed human glycosylasparaginase in insect Sf9 cells*

Spinner cultures of Sf9 cells were infected with either wild-type or recombinant Ac[mel-GSA] baculovirus and samples of the media collected at 24 h intervals. Immunoblot analysis demonstrated Sf9 cells harboring recombinant Ac[mel-GSA] baculovirus secreted two cross-reacting peptides, 41 and 26 kDa, at the sampling time points 24, 48, and 72 hpi, with a third peptide of 23 kDa appearing at 72 hpi (Fig. 1). These three bands represent the precursor glycosylasparaginase protein (41 kDa) and two heavy subunit variants (26 kDa and 23 kDa). By 120 hpi only the 23 kDa heavy subunit was evident. Enzyme assays revealed recombinant glycosylasparaginase secreted from Ac[mel-GSA] baculovirus infected Sf9 cells was catalytically active (Fig. 1). The quantity of enzyme activity secreted into the media increased from 24 to 120 hpi in apparent agreement with the increase in immunoblot band intensities over the same period. There was no detectable GlcNAc-Asn cleaving activity in the media collected from Sf9 cells infected with wild-type baculovirus. At 72 hpi when the media was harvested the total production of secreted glycosylasparaginase enzyme was 430 milliunits or 172 μg protein (protein value based on the specific activity of purified glycosylasparaginase being 2.5 units/mg) from a 100 ml spinner culture.

A sample of the media was treated with *N*-glycanase revealing a deglycosylated 40 kDa precursor and processed heavy subunits of 25 kDa and 22 kDa (Fig. 2). The

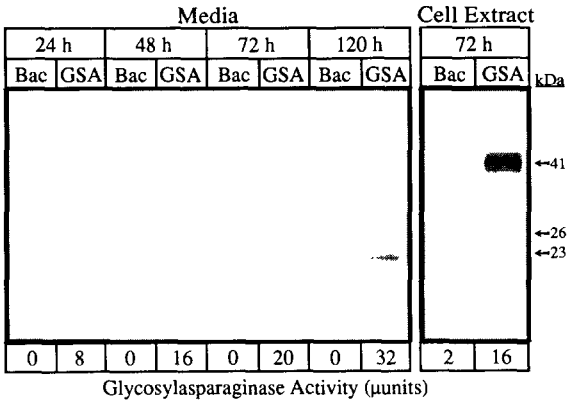

Fig. 1. Time-course analysis of secreted and intracellular human glycosylasparaginase expressed from a recombinant baculovirus. Sf9 cells were infected with Ac[mel-GSA] (lanes labeled *GSA*) or wild-type (lanes labeled *Bac*) baculovirus and media samples collected at 24 h intervals. An extract of the infected cells was prepared at 72 hpi. Samples were resolved on a 15% polyacrylamide gel, electroblotted onto a poly(vinylidene difluoride) (PVDF) (Immobilon; Millipore) membrane, and incubated with antibodies against the heavy subunit of glycosylasparaginase. The approximate molecular mass of the single- chain precursor and two heavy subunit isoforms are indicated The glycosylasparaginase activity detected in media and cell extract samples is given below each sample. One unit of activity was defined as the quantity of enzyme that released one μmole of *N*-acetylglucosamine/min at 37°C.

two heavy subunits are therefore proteolytic isoforms that arise during maturation of the single-chain glycosylasparaginase precursor. The first clippage likely occurs between residues Asp-205–Thr-206 resulting in the light subunit and the 26 kDa pre-form of the heavy subunit. This is followed by hydrolysis of a second peptide bond in the heavy subunit that generates the fully processed 23 kDa peptide. The disappearance of both the 26 kDa heavy subunit isoform and the 41 kDa single-chain precursor from the media by 120 hpi suggests the involvement of in vitro processing by cellular factors that were released into the media during baculovirus infection. Vernet et al. [21] observed a similar conversion of a recombinant papain zymogen secreted from Sf9 cells to a smaller molecular mass active peptide. Glycosylasparaginase heavy subunit processing appears to have little significance in terms of catalytic potential since media samples with only the 26 kDa isoform (24 hpi and 48 hpi) or the fully processed 23 kDa peptide (120 hpi) both displayed activity (Fig. 1). Antibodies specific for the light subunit of glycosylasparaginase revealed the secreted protein from Ac[mel-GSA] infected cells contains two light subunit variants of 17 kDa and 18 kDa (Fig. 2). Unlike the two heavy subunits, the light subunit isoforms are due to differential glycosylation since the two are converted to a single 15 kDa band after removal of oligosaccharides. The processing of recombinant glycosylasparaginase expressed from insect cells closely mirrors the post-translational modifications that occur in mammalian cells. We have shown previously that COS-1 cells transiently transfected with a human placenta glycosylasparaginase cDNA express a 43 kDa precursor polypeptide that is post-translationally processed resulting in two heavy subunits (27 kDa and 24 kDa) and two light subunits (17 kDa and 18 kDa) [8].

Although the honeybee melittin signal peptide was capable of directing catalytically active glycosylasparaginase outside the cell, an extract of Ac[mel-GSA] infected cells prepared 72 hpi revealed most of the recombinant protein that was expressed remained intracellular in the form of single-chain precursors 40, 41, and 42 kDa (Fig. 1). The 26 and 23 kDa processed heavy subunits and several minor proteolytic fragments were also evident in the cell extract but at substantially lower levels. None of the glycosylasparaginase peptides detected with the heavy subunit-specific antibodies were expressed by cells infected with wild-type baculovirus (Fig. 1). The intracellular glycosylasparaginase activity was determined and found to be 8-fold greater than Sf9 cells infected with wild-type virus (Fig. 1). This amounted to 140 milliunits or 56 μg recombinant protein from the 100 ml spinner culture cell pellet. Therefore, while the concentration of immunoreactive protein appeared greatest intracellularly, there was three times more activity in the media. The protein extract from Ac[mel-GSA] infected cells was resolved on a gel filtration column in an attempt to separate the glycosylasparaginase single-chain precursor from the mature subunit conformation. An immunoblot of the column fractions indicated the protein peak which corresponded to the

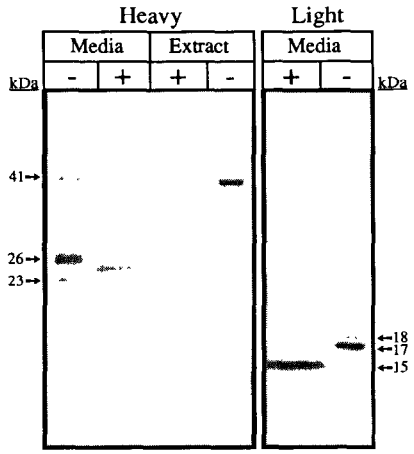

Fig. 2. *N*-Glycase treatment of human glycosylasparaginase expressed in Sf9 insect cells. An extract of Ac[mel-GSA] infected cells was prepared 72 hpi and resolved on a Sephacryl S-200 gel filtration column. Fractions devoid of the processed subunits were pooled and treated with *N*-glycanase to remove asparagine-linked oligosaccharides. A sample of the media harvested at 72 hpi was also incubated with *N*-glycanase. Extract and media samples before (labeled −) and after (labeled +) *N*-glycanase treatment were electrophoresed on a 15% SDS-polyacrylamide gel, blotted onto a PVDF membrane, and incubated with glycosylasparaginase heavy or light subunit-specific antibodies as indicated. The approximate molecular mass of the precursor and processed peptides before deglycosylation are given.

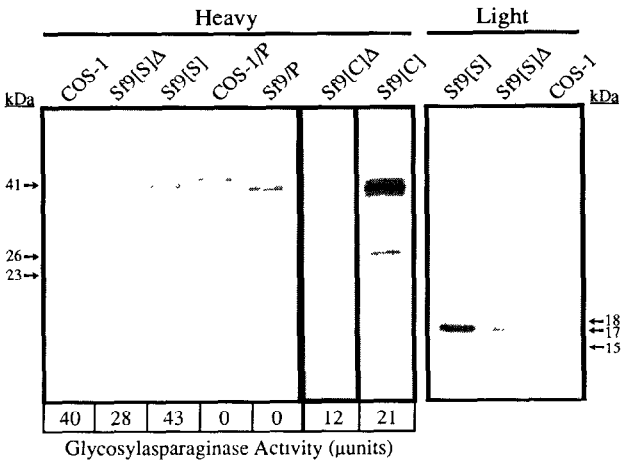

Fig. 3. Catalytic activity of precursor and processed glycosylasparaginase expressed in insect Sf9 cells and mammalian COS-1 cells. Blots were incubated with antibodies against the heavy or light subunits of glycosylasparaginase as indicated. Each sample's corresponding glycosylasparaginase activity is given. Lanes labeled *COS-1* show recombinant glycosylasparaginase expressed in COS-1 cells transiently transfected with a pSVL vector containing a human placenta glycosylasparaginase cDNA [8]. A sample of the single-chain precursor from COS-1 cells without the processed subunits is also shown (lane labeled *COS-1/P*) This protein was generated from a pSVL vector containing a glycosylasparaginase cDNA sequence that encodes the Finnish AGU G488 → C point mutation [8] Lanes labeled *Sf9[S]* contain recombinant glycosylasparaginase secreted from Ac[mel-GSA] infected Sf9 cells, while lanes labeled *Sf9[S]Δ* show an aliquot of the same material heated at 65°C for 15 min. Samples of intracellular recombinant glycosylasparaginase from Ac[mel-GSA] infected Sf9 cells before (lane labeled *Sf9[C]*) and after (lane labeled *Sf9[C]Δ*) heat treatment at 65°C are also shown The intracellular precursor from Ac[mel-GSA] infected Sf9 cells following gel filtration chromatography appears in the lane indicated *Sf9/P* The approximate molecular masses of the glycosylasparaginase peptides expressed in Sf9 cells are shown.

precursor eluted with a slightly earlier retention time compared to the processed form, resulting in several early fractions that were free of the glycosylasparaginase subunits (data not shown). These fractions were pooled, subject to *N*-glycanase digestion, and visualized with heavy subunit-specific antibodies. Removal of asparagine-linked oligosaccharides resulted in the same 40 kDa band that was detected in the deglycosylated media sample (Fig. 2). The three intracellular single-chain forms that were present before digestion therefore represent the precursor polypeptide lacking asparagine-linked sugars (40 kDa) and two carbohydrate variants (41 kDa and 42 kDa).

Having obtained a preparation of the glycosylasparaginase precursor absent of the processed subunits, an enzyme assay was performed to determine its catalytic potential. No detectable release of free glucosamine from the GlcNAc-Asn substrate was observed (Fig. 3). This indicated that the recombinant activity detected in the extract and media from cells infected with the Ac[mel-GSA] baculovirus was due to the processed subunit conformation. However, to avoid any ambiguity we sought to remove the single chain precursor from the media and cell extracts that were harvested at 72 hpi. This was accomplished by taking advantage of the heat stability conferred by the native glycosylasparaginase conformation [3,22]. After heating the sample and removing any denatured material by centrifugation, immunoblotting revealed the precursor had been precipitated while the majority of the processed heavy and light subunits remained soluble (Fig. 3). The heat treated sample also retained 65% of its enzyme activity. The selective removal of the precursor polypeptide from the processed active enzyme was further shown with a sample from the intracellular extract (Fig. 3). The contrasting data showing the precursor is catalytically inactive while the processed heavy-light subunit organization is functional provide convincing evidence that glycosylasparaginase is a zymogen (Fig. 3). Samples of the precursor and processed formes of glycosylasparaginase expressed in COS-1 cells have been included in Fig. 3 along with those from Sf9 cells to demonstrate their similarity in processing and associated activities.

3.2. *Mutagenesis of Thr-206 destroys glycosylasparaginase activity*

Two different glycosylasparaginase cDNAs were constructed by in vitro mutagenesis to determine the importance of Thr-206 for catalysis. The first encoded a Thr-206 → Ala substitution at amino acid position 206 ($GSA^{Ala\text{-}206}$) and the second a Thr-206 → Ser substitution ($GSA^{Ser\text{-}206}$). Expression in COS-1 cells revealed neither of the two substitution mutants produced an increase in GlcNAc-Asn cleaving activity above mock transfected cells (Fig. 4). This result was in contrast to cells transfected with the normal human placenta glyco-

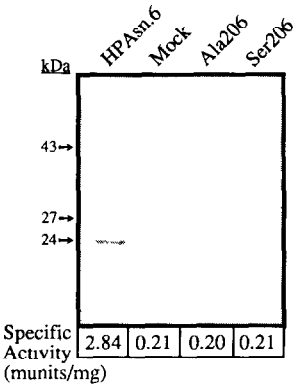

Fig. 4. Expression of glycosylasparaginase active site mutants in COS-1 cells. Lysates from cells transfected with the human placenta cDNA HPAsn.6 [4], $GSA^{Ala\text{-}206}$, $GSA^{Ser\text{-}206}$, or mock-transfected with the native pSVL expression vector were resolved on a 15% SDS-polyacrylamide gel and electrophoretically transferred to a PVDF membrane. The blot was incubated with heavy subunit-specific antibodies. Aliquots of the same lysates used for immunoblot analysis were also assayed for glycosylasparaginase activity. The specific activity of each lysate is given in milliunits/mg (nanomoles *N*-acetylglucosamine released/min/mg protein at 37°C).

sylasparaginase cDNA HPAsn.6 where a 13-fold increase in the release of free glucosamine was observed. The presence of recombinant glycosylasparaginase in the transfected COS-1 cell lysates was demonstrated by immunoblot analysis with antibodies specific for the heavy subunit. The fully processed 24 kDa heavy subunit was found to be the predominant immunoreactive peptide in COS-1 cells expressing the human placenta cDNA HPAsn.6, with minor quantities of the 27 kDa heavy subunit pre-form and 43 kDa precursor polypeptide also present (Fig. 4). None of the glycosylasparaginase specific bands were detected in mock-transfected cells (Fig. 4). COS-1 cells transfected with either of the glycosylasparaginase substitution mutants contained the 24 kDa heavy subunit revealing the lack of enzyme activity was not due to the absence of recombinant protein (Fig. 4). Interestingly, a significant quantity of the 43 kDa glycosylasparaginase precursor accumulated in cells transfected with the $GSA^{Ala-206}$ cDNA. This inhibitory effect on subunit processing likely contributes to the absence of catalytic function.

According to the glycosylasparaginase reaction mechanism as proposed by Kaartinen et al. [16], the first of three symmetrical steps involves the formation of a β-aspartyl acyl-enzyme intermediate mediated through the hydroxyl group of Thr-206. Therefore, it would seem reasonable to suggest that the inactive state of the Ala-206 mutant is due in part to the lack of a nucleophilic hydroxyl moiety. This same reasoning cannot be applied to the Ser-206 mutant. One possible explanation for the absence of glycosylasparaginase activity is that the Ser-206 substitution interferes with correct subunit processing. In this instance the heavy and light subunits are formed (Fig. 4), however, post-translational processing fails to place Ser-206 at the newly formed amino terminus of the light subunit. Proteolytic cleavage at a nearby cryptic site is one model where subunit processing could occur without yielding an amino terminal Ser-206. Although the inability to exchange Ser-206 for Thr-206 without compromising glycosylasparaginase catalytically activity is difficult to rationalize in the absence of refined structural data, it is of interest that there appears to be a tight evolutionary conservation for an active site threonine residue among mammalian [23], bacterial [24], yeast [25] and plant [26] amidohydrolases.

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Note added in proof

Recently Ikonen et al. [27] using site-specific mutagenesis have also shown that conversion of the single chain form of glycosylasparaginase into its heavy and light chains is an activation step that occurs prior to the lysosomes.